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In-house quantitative real-time PCR for the diagnosis of hepatitis B virus and hepatitis C virus infections

Danielle Alves Gomes Zauli[a,*], *Carla Lisandre Paula de Menezes*[b], *Cristiane Lommez de Oliveira*[a], *Elvis Cristian Cueva Mateo*[a], *Alessandro Clayton de Souza Ferreira*[a]

[a] *Instituto Hermes Pardini, Departamento de Pesquisa e Desenvolvimento, Vespasiano, MG, Brazil*
[b] *Linhagen Produtos em Biotecnologia Ltda, Belo Horizonte, Minas Gerais, Brazil*

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ABSTRACT

The quantification of viral nucleic acids in serum by real-time PCR plays an important role in diagnosing hepatitis B virus and hepatitis C virus infection. In this study, we developed an assay using specific primers and probes to quantify hepatitis B virus DNA or hepatitis C virus RNA in serum from infected patients. For standardization and validation of the assay, an international panel of hepatitis B virus/hepatitis C virus and standard plasmids was used. A correlation coefficient of 0.983 and 0.963 for hepatitis B virus and hepatitis C virus, respectively, was obtained based on cycle threshold values and concentrations of DNA or RNA. The standard curve showed a linear relationship from 19 IU/mL to 1.9×10^9 IU/mL of serum, with a coefficient of determination (r^2) of 0.99. In sera from patients infected with hepatitis B virus or hepatitis C virus viral loads (19 IU/mL and 1.9×10^9 IU/mL), we quantified viral loads with a detection limit of 1.9×10^2 IU/mL. The real-time quantitative PCR assay developed in this study provides an ideal system for routine diagnosis and confirmation of indeterminate serological results, especially in immunosuppressed patients.

Introduction

Acute and chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) lead to significant mortality and are a major public health problem worldwide. According to the World Health Organization (WHO), 130–150 million people are infected with HCV worldwide and 240 million people are chronically infected with HBV. An estimated 30% of people infected develop liver cirrhosis and/or hepatocellular carcinoma (HCC).[1–3]

HBV and HCV infection is usually diagnosed by the detection of anti-HBV/or anti-HCV antibodies in a patient's serum that react to recombinant HBV or HCV proteins in an enzyme immunoassay or chemiluminescence immunoassay. However, these markers have limitations that reduce diagnostic accuracy. In order to overcome these problems, several assays have been developed in the last few years to detect nucleic acids of hepatitis-causing viruses.[4–8]

Determining HBV DNA or HCV RNA levels in serum has become an important tool to identify individuals with high viral loads, which may suggest high infectivity, to monitor disease progression and the efficacy of antiviral therapies, to detect drug resistant mutants, and to identify relapse after the discontinuation of an antiviral therapy.[9–12] A limitation in hepatitis virus research is the lack of a sensitive and highly specific test to measure viral loads in plasma or serum. Previous studies have used real-time PCR-based approaches to determine viral loads in infected patients. With high sensitivity and specificity, broad detection capability, simplicity, and reproducibility, this technique is particularly useful for the analysis of a large number of specimens and to measure viral load.[13–16] In the present study, we developed a sensitive method to quantify HBV DNA and HCV RNA in sera from infected patients by quantitative real-time PCR (qPCR) using specific primers and TaqMan minor groove binding fluorescent probe technology.

Materials and methods

Patients and serum samples

A total of 116 serum samples were obtained from patients infected with HBV or HCV. This study was approved by the Ethics Committee of Universidade Federal de Juiz de Fora (protocol 042/2010).

Selection and design of primers and probes for quantitative real-time PCR

The sequences of the primer and probes used in this study are listed in Table 1. The primers were selected from published literature, and the probes were designed using Primer Express Software (Applied Biosystems, Foster, CA, USA). Programs, including AmplifX (CNRS, Aix-Marseille Université, http://crn2m.univ-mrs.fr/pub/amplifx-dist), were used to predict the behavior of these primers. The expected hybridization and specificity of primer–probe sets targeting HBV DNA and HCV RNA were determined by in silico analysis using the BLAST program (http://ncbi.nlm.nih.gov/BLAST). The reporter dye FAM was attached to the 5′ ends of probes, and a non-fluorescent quencher (NFQ) and minor groove binder (MGB) were attached to the 3′ ends. All primers and probes were synthesized by Integrated DNA Technologies.

Extraction of nucleic acids

Nucleic acids were extracted from 200 μL of serum using a QIAamp MinElute Virus Spin kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's suggested protocol. The concentration and quality of the extracted DNA and RNA were assessed by Nanovue spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA) and by amplification of a fragment of the gene coding for β-actin (internal control) (GenBank No. AY5827991). The extracted DNA and RNA were stored at −80 °C until use.

cDNA synthesis

Viral RNA was reverse transcribed using random primers and a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's suggested protocol. The resulting cDNA was stored at temperature −20 °C until use.

Conventional PCR

Conventional PCR was used to evaluate the sizes of amplified products. The amplification conditions were the following: a 10-μL reaction mixture containing 50–100 ng/L template DNA, 0.1 μmol/L primer mixture, 1 μmol/L deoxynucleoside triphosphates, 1 μL of 10× buffer, 2 mmol/L MgCl$_2$, and 0.05 μL Gold Star Taq polymerase, with cycling conditions that included an initial denaturation and polymerase activation step for 10 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 60 °C for 1 min, extension at 72 °C for 2 min, and then a final extension step of 10 min at 72 °C. All PCR products were visualized following 6% polyacrylamide gel electrophoresis and staining of gels with silver nitrate as described by Ref. 19.

Table 1 – Primers and probes used in this study.

Virus	Gene	Sequences of primers and probes (5′–3′)	Amplicon size (base pairs)	Reference
HBV	S gene	[a]FW: CCTGGYTATCGYTGGATGTGT [b]RV: GGACAVACGKGCAACATACCTT Probe: 6FAM CTATGCCTCATCTTCTT MGB NFQ	116	17
HCV	Non-coding region	FW: AGCGTCTAGCCATGGCGTT RV: GCAAGCACCCTATCAGGCAGT Probe: FAM TCTGCGGAACCGGTGAGT MGB NFQ	238	18

[a] Forward primer.
[b] Reverse primer.

Optimization of the qPCR assay

The qPCR assay was optimized following the MIQE guidelines.[20] Primer and probe matrix experiments were conducted by selecting, for each gene, the primer concentration that resulted in the lowest cycle threshold (Ct) and the highest ΔRn using a fixed amount of target template. Reactions with different concentrations of primers and probes were performed in total volumes of 25 μL and 12.5 μL, of TaqMan Universal Master Mix buffer (Applied Biosystems, Foster CA, USA); sense primer and antisense primer concentrations of 50 nM, 300 nM, and 900 nM each; probes at concentrations of 80 nM, 125 nM, 150 nM, and 250 nM; and 1 μL of DNA or cDNA. The reaction was performed with a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using universal conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. For these reactions, an OptiQuant HBV/HCV Quantification Panel (AcroMetrix, Benicia, CA, USA) was used in the following concentrations expressed in international units/mL (IU/mL): HBV 7 or HCV 7 (5,000,000 IU/mL) and HBV 2 or HCV 2 (50 IU/mL).

Validation of the assay with international standards

For validation of the assay, the international panel cited above was used in qPCR reactions to construct standard curves of HBV DNA or HCV RNA in the following concentrations: HBV 7 or HCV 7 (5,000,000 IU/mL), HCV 6 or HBV 6 (500,000 IU/mL), HBV 5 or HCV 5 (50,000 IU/mL), HBV 4 or HCV 4 (5000 IU/mL), HBV 3 or HCV 3 (500 IU/mL), and HBV 2 or HCV 2 (50 IU/mL). The reactions were performed in a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the TaqMan detection system (Thermo Fisher Scientific, Waltham, MA, USA) with 12.5 μL of TaqMan Universal Master Buffer, predetermined concentrations of the primer–probe sets cited above, and 50 - 100ng of DNA or cDNA, for a total final volume of 25 μL per reaction. All reactions were performed in duplicate using universal conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Results were analyzed using 7500 Fast Software v. 2.1 (Life Technologies, Carlsbad, CA, USA) and expressed in IU/mL. The baseline and threshold values were automatically adjusted for each test.

Quantification of viral loads

To quantify the viral loads, standard curves were constructed from a dilution series of cloned plasmids containing inserts in the conserved region of each virus in initial concentrations of 5.1×10^9 IU/mL and 4.9×10^9 IU/mL for HBV and HCV, respectively, with a total of eight points in each curve. The fragments were amplified by conventional PCR and cloned using a TOPO TA Cloning Kit (Invitrogen, California, CA, USA) according to manufacturer's instructions. The cloning and transformations were performed using a CloneJET PCR Cloning Kit and TransformAid Bacterial Transformation Kit, respectively (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The concentration of recombinant plasmids was determined using a Qubit 2.0 Fluorometer (Thermo Fisher

Scientific, Waltham, MA, USA), and reactions were performed in a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the TaqMan detection system (Life Technologies, Carlsbad, CA, USA). Assays consisted of 12.5 μL of TaqMan Universal Master Buffer, predetermined concentrations of primers and probes, and 50–100 ng of DNA or cDNA, for a final volume of 25 μL per reaction. All reactions were performed in duplicate. Results were analyzed using 7500 Fast Software v. 2.1 (Thermo Fisher Scientific, Waltham, MA, USA) and expressed in IU/mL of the target template. The baseline and threshold values were automatically adjusted for each test. The relative efficiency of amplification was assessed by the slope of a linear regression of Ct values against concentrations of DNA or cDNA. The efficiency of the reaction was determined according to the following formula[20]:

$$E = \left(10 \left(\frac{-1}{\text{slope}} \right) - 1 \right) \times 100.$$

Validation of the assay with serum samples

To validate this assay, serum samples were collected from patients infected, either acutely or chronically, with HBV or HCV and maintained at −20 °C until use. The inclusion criterion was the presence of HBV or HCV infection. The exclusion criterion was the absence of HBV or HCV infection. This set of 49 HBV and 67 HCV positive sera were used to validate the qPCR assay. For the qPCR reactions, a dilution series of the standard plasmid was used as the standard curve.

Limit of detection (LOD)

The LOD of the assay was determined by serially diluting the standard plasmid (1.9×10^{10}–0.19×10^0 IU/mL) and identifying the lowest concentration of each clone yielding a 90% or greater detection rate.

Results

Conventional PCR

The sizes of the products amplified by conventional PCR are presented in Fig. 1. Products were of the expected size, indicating amplification of the target template.

Standardization of qPCR

In order to standardize the qPCR assay for quantification of HBV or HCV, different primer and probe concentrations were used to determine the optimal detection conditions. Table 2 shows the Ct values and slope of the linear regression from reactions at these concentrations. Using the HBV 7 or HCV 7 controls as templates, amplification was detected in all combinations of primer sets. For the HBV 2 or HCV 2 controls, amplification was detected only at forward and reverse primer concentrations of 50 nM and 300 nM, respectively. We found no difference in assay performance at the various probe concentrations, and a final probe concentration of 125 nM was used in all qPCR reactions.
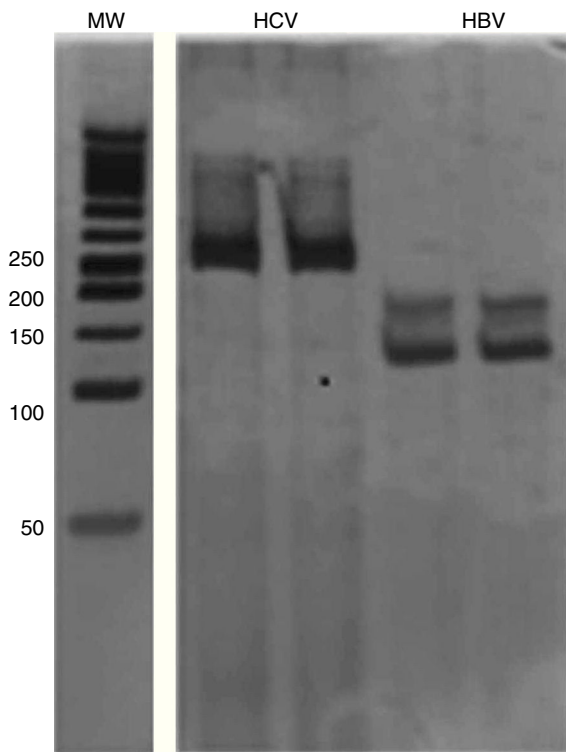
Fig. 1 – Gel electrophoresis (6% polyacrylamide) of products amplified from HBV and HCV coding genes by conventional PCR. MW, molecular weight standard (50 bp).

International standard	Primer concentration FW/RV	Ct value	Slope value
HBV 7[a]	50 nM/50 nM	28.86785507	−1.0434
	50 nM/300 nM	26.90498924	
	50 nM/900 nM	26.2933445	
	300 nM/50 nM	25.48120117	
	300 nM/300 nM	23.62826729	
	300 nM/900 nM	23.69248581	
HBV 2[b]	50 nM/50 nM	37.58005905	1.4634
	50 nM/300 nM	39.04344177	
	50 nM/900 nM	Undetermined	
	300 nM/50 nM	Undetermined	
	300 nM/300 nM	Undetermined	
	300 nM/900 nM	Undetermined	
HCV 7[a]	50 nM/50 nM	29.46348	0.7627
	50 nM/300 nM	29.3045578	
	50 nM/900 nM	28.48422432	
	300 nM/50 nM	28.22242737	
	300 nM/300 nM	31.08761597	
	300 nM/900 nM	33.78520966	
HCV 2[b]	50 nM/50 nM	Undetermined	Undetermined
	50 nM/300 nM	36.86901855	
	50 nM/900 nM	Undetermined	
	300 nM/50 nM	Undetermined	
	300 nM/300 nM	Undetermined	
	300 nM/900 nM	Undetermined	

Table 2 – Results (Ct values and slope) from the qPCR reactions using different concentrations of primer–probe sets targeting conserved HBV and HCV genes.

[a] Viral load: 5,000,000 IU/mL (HBV 7/HCV 7).
[b] Viral load: 50 IU/mL (HBV 2/HCV 2).

Validation of the qPCR assay with international standards

To validate the assay for quantitation of HBV and HCV, a standard curve was constructed using HBV DNA or HCV RNA from an international panel. The amplification plots and standard curves of both viruses are shown in Fig. 2. Regressions of the Ct values and concentrations of HBV DNA or HCV cDNA showed correlation coefficients of 0.983 and 0.963 for HBV and HCV, respectively. The slopes of the standard curves for HBV and HCV were −3.438 and −2.898, respectively.

Quantification of viral loads

In order to obtain a standard curve for absolute quantitation of HBV DNA and HCV RNA, 10-fold dilutions of standard plasmids were used as templates. The amplification plot and standard curve (Fig. 3) that were generated showed a linear relationship from 5.1×10^2 to 5.1×10^9 IU/mL and 4.9×10^2 to 4.9×10^9 IU/mL for HBV and HCV, respectively. The slope values were −3.583 and −3.263 for HBV and HCV, respectively. Linear regression analysis yielded a coefficient of determination (r^2) of 0.99 and efficiencies of 90–100% for both HBV and HCV.

Validation of the assay with serum samples

A total of 49 serum samples from patients infected with HBV and 67 serum samples of patients infected with HCV were analyzed by qPCR. In all clinical samples, it was possible to quantify the viral load and optimize the assay for acceptable sensitivity.

LOD of the assay

A dilution series of standard plasmids for each virus, ranging from 1.9×10^{10} to 0.19×10^0 IU/mL, was used to determine the LOD of the assay. The 90% LOD for the assay was found to be 1.9×10^2 IU/mL for each virus.

Discussion

In this study, we developed an in-house qPCR-based assay for the quantification of HBV DNA and HCV RNA in sera from infected patients. The assay, proposed for use in diagnosis of HBV and HCV, amplified conserved regions of the HBV and HCV genomes and was standardized and validated using different concentrations of primer–probe sets and international nucleic acid standards. Viral DNA or RNA, isolated by a commonly used nucleic acid extraction kit, could be used to quantify virus in an initial sample volume of only 200 μL.

The primers used for HBV detection are specific for a highly conserved region of the S gene.[17] All HBV genotypes found in Brazilian patients and described in previous studies were detected with the same primers used in this study.[17,21] The primers selected for HCV target the conserved 5′ non-translated region of the viral RNA. According to the literature, all genotypes of HCV can be detected with these primers.[18]
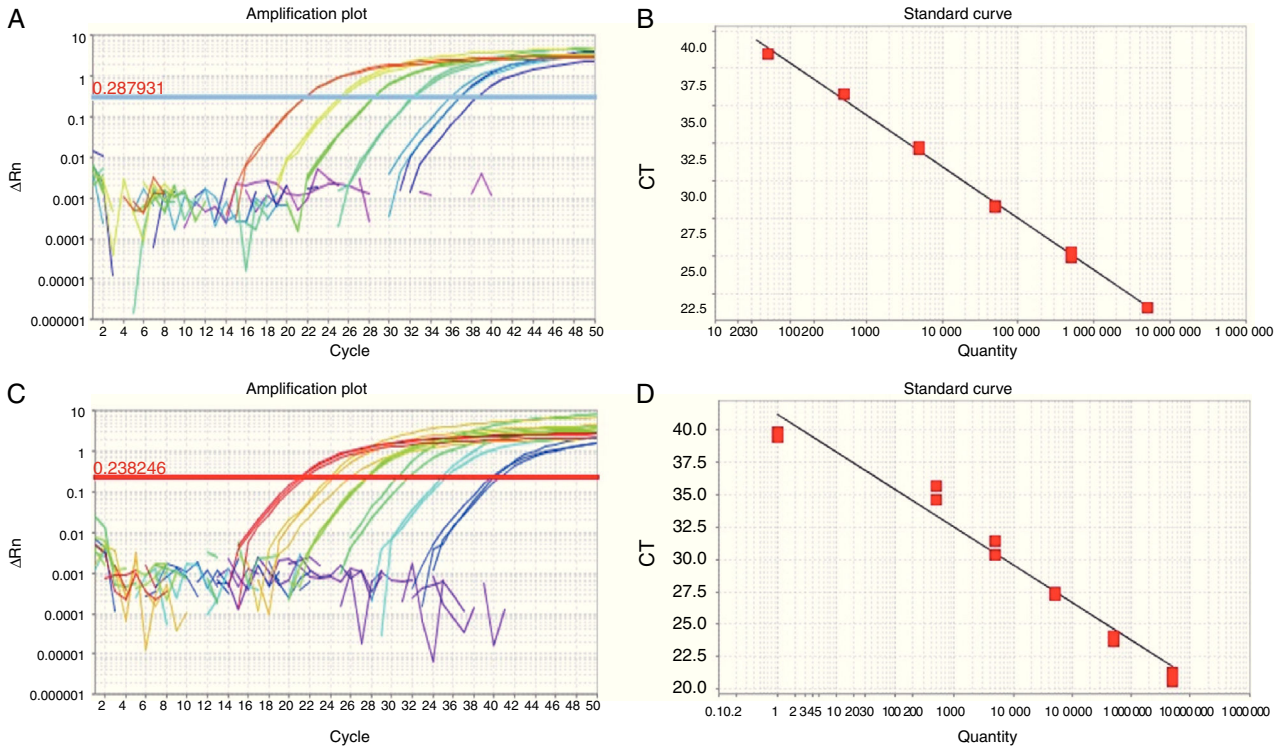
Fig. 2 – Amplification plot and standard curve of HBV (A and B, respectively) and HCV (C and D, respectively) obtained using the International OptiQuant HBV/HCV Quantification Panel (AcroMetrix, Benicia, CA, USA) to validate the qPCR assay. The results are expressed in international units/mL (IU/mL).
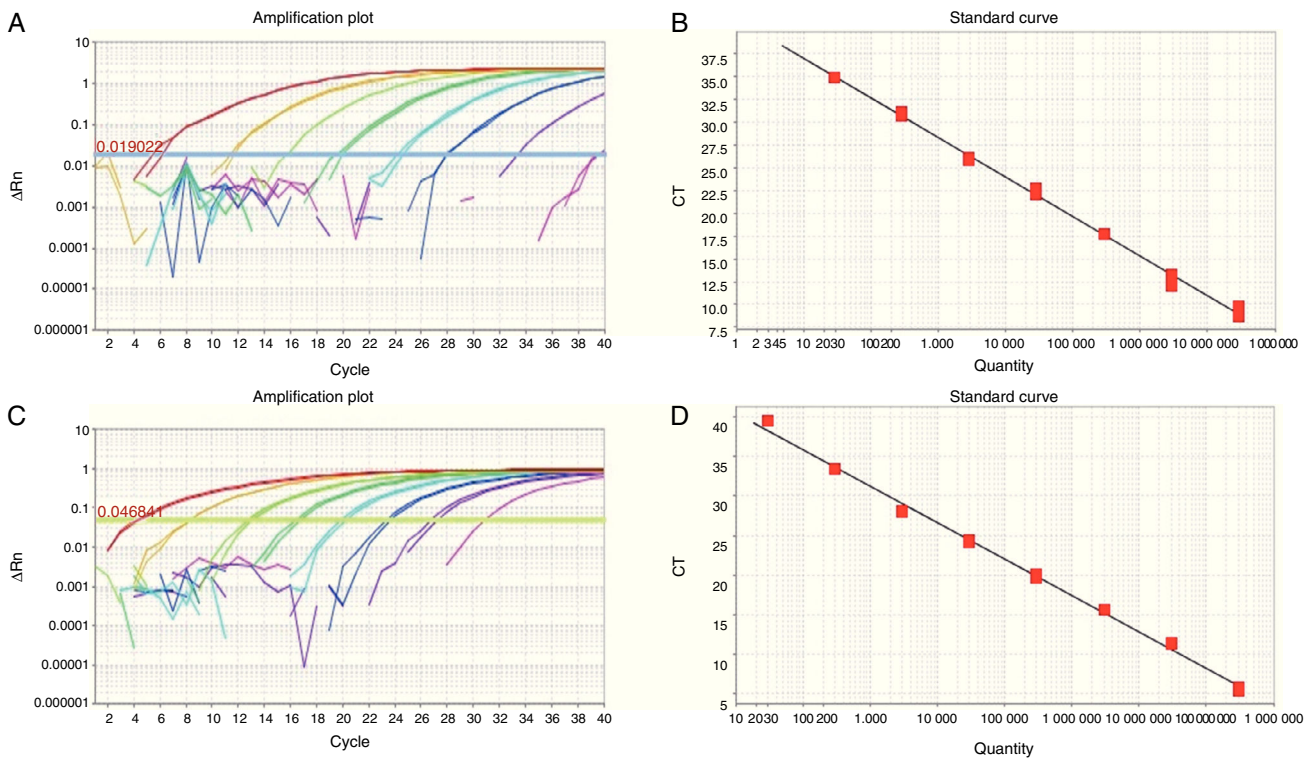
Fig. 3 – Amplification plots and standard curves prepared using standard plasmids for quantification of HBV (A and B, respectively) and HCV (C and D, respectively) viral loads. The dilution series of the standard plasmids of HBV DNA and HCV RNA began at 5.1×10^9 IU/mL and 4.9×10^9 IU/mL for HBV and HCV, respectively. The results are expressed in IU/mL.

Moreover, the 5′ nuclease fluorogenic probe used in this study has greater specificity and sensitivity and a broader dynamic range of detection than assays that use DNA binding dyes. Therefore, the assay should allow accurate quantification of all HBV and HCV genotypes.

In this study, the qPCR assay detected loads of HBV DNA ranging from 5.1×10^9 to 5.1×10^2 IU/mL and HCV RNA ranging from 4.9×10^9 to 4.9×10^2 IU/mL in serum. Furthermore, the assay efficiently quantified both viruses ($r^2 = 0.99$), with an adequate LOD (1.9×10^2 IU/mL) for an in-house qPCR. To demonstrate the effectiveness of the qPCR assay proposed in this study, clinical samples from patients infected with HBV or HCV were tested. The results suggest that the assay can be used to quantify both low and high viral loads and that its amplification efficiency is stable over a range of input copy numbers.

It is important to note that comparison of results from this assay with those from currently recommended clinical protocols are needed before this assay can be used for long-term monitoring of HBV and HCV treatments. In conclusion, in this study we developed a novel qPCR assay, based on the TaqMan MGB system, that is rapid, sensitive, and accurate. This assay, validated with both international standards and clinical samples, provides an ideal system for routine diagnosis, to confirm indeterminate serological results, especially in immunosuppressed patients, and to detect viremia prior to seroconversion.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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